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Production Methods for a Mesenchymal Stem Cell Therapeutic as a Medical Defense Countermeasure

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14. ABSTRACT
The application of cellular therapies to treat battlefield injuries offers a novel and promising approach to address longstanding challenges in the repair of tissue damage with regard to both structural and functional improvement. The results of currently published investigations describing mesenchymal stem cell (MSC) efficacy in a variety of injury models demonstrate the unique qualities of this reparative cell population to adapt to the requirements of the damaged tissue in which the cells integrate. MSC therapy represents a single medical intervention that can simultaneously provide a broad range of therapeutic efficacy, with local activity, at multiple tissue and organ sites. The data presented in this report demonstrate that MSCs can be routinely isolated from adult bone marrow and expanded in cell culture to consistently and reliably produce pharmaceutical quantities of therapeutic product. Characterization of stem cell properties of culture-expanded MSCs is shown by *in vitro* differentiation to form mature cell types. The production methodology described will provide the means for producing test material for use in evaluating MSC therapy as a candidate treatment modality for injuries of concern to medical defense.

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SUMMARY

The application of cellular therapies to treat battlefield injuries offers a novel and promising approach to address longstanding challenges in the repair of tissue damage with regard to both structural and functional improvement. The results of currently published investigations describing mesenchymal stem cell (MSC) efficacy in a variety of injury models demonstrate the unique qualities of this reparative cell population to adapt to the requirements of the damaged tissue in which the cells integrate. MSC therapy represents a single medical intervention that can simultaneously provide a broad range of therapeutic efficacy, with local activity, at multiple tissue and organ sites.

The data presented in this report demonstrate that MSCs can be routinely isolated from adult bone marrow and expanded in cell culture to consistently and reliably produce pharmaceutical quantities of therapeutic product. Characterization of stem cell properties of culture-expanded MSCs is shown by *in vitro* differentiation to form mature cell types. The production methodology described will provide the means for producing test material for use in evaluating MSC therapy as a candidate treatment modality for injuries of concern to medical defense.

INTRODUCTION

Intravenously (IV) infused MSCs have been shown to specifically home to sites of tissue damage in multiple preclinical injury models.[1-3] MSC infusion mimics a naturally occurring process in which endogenous MSCs leave the bone marrow compartment in response to injury, enter the circulation, and travel to sites of tissue damage due to the influence of chemotactic homing signals released at each compromised site. Clinical development of MSC formulations for therapeutic use has involved the isolation of MSCs from bone marrow and expansion in culture.[4] Numerous studies have shown that donor-derived, IV-administered MSCs retain the ability to home to damaged tissue and facilitate repair in a variety of injury and disease settings. Cell culture-expanded MSCs demonstrate the potential to form several specialized cell types, including cardiac and skeletal muscle, lung and kidney epithelium, skin, bone, fat, cartilage, tendon, and many others. Once engrafted within damaged tissue, MSCs participate in the healing process both directly, through differentiation to replace lost cell types, and indirectly, through the local secretion of cytokines and other bioactive molecules that facilitate a reduction in inflammation, inhibition of scar formation, and the enhancement of endogenous mechanisms of tissue reconstruction.[5]

The molecular basis for MSC homing to injury sites has been evaluated by several independent laboratories using both *in vitro* and *in vivo* approaches. These data describe MSC chemotaxis toward a variety of chemokines, including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), interleukin-8 (IL-8), and stromal derived factor-1 (SDF-1).[6-8] For example, Wang and colleagues (2002)[6] observed *in vitro* MSC migration toward purified MCP-1, MIP-1α and IL-8, as well as toward extracts prepared from brain tissue injured by oxygen deprivation (ischemic injury). Antibodies against MCP-1 significantly inhibited MSC migration toward damaged brain tissue, suggesting a prominent role for this chemokine in MSC recruitment.[9] Local expression of the chemokine SDF-1 has also been shown to play a critical role in MSC homing to injured brain tissue, as well as to tissue damage in several other organs. Wang *et al.* (2008)[10] have shown that pre-infusion incubation of MSCs with AMD3100, an antagonist of the SDF-1 receptor CXCR4, prevents MSC migration to cerebral injury. This work is supported by observations that intracerebral injection of SDF-1 stimulates MSC trafficking to the site of injection.[11] In a rat model of ischemic heart injury, local SDF-1 expression is transiently upregulated. Although expression of the endogenous chemokine declines to nearly undetectable levels within 7 days, MSC homing to the site of injury can be restored at time points as late as 8 weeks post-injury by intra-lesion transplantation of cardiac fibroblasts engineered to express and secrete SDF-1.[7]

Once engrafted to damaged tissue, MSCs elicit a broad range of effects with regard to modulation of the inflammatory response to injury. MSCs express a low level of major histocompatibility (MHC) class I molecules, but lack expression of MHC class II and the B7 co-stimulatory molecule. The cells therefore evade recognition by both $CD4^+$ T helper and $CD8^+$ cytotoxic T cells. Cell surface markers of lymphocyte activation, including CD25, CD38, and CD69, have been shown to decrease in the presence of

MSCs. T cell proliferation is inhibited by MSCs through a block in cyclin D2 expression, resulting in cell cycle arrest. Finally, MSCs have also been shown to inhibit the innate immune response by blocking IL-2–mediated activation of natural killer cells (reviewed in Newman *et al.*, 2009[12] and Paul *et al.*, 2009[13]).

In addition to the initial anti-inflammatory properties of MSCs, engraftment results in the local secretion of a variety of paracrine factors that facilitate wound healing. These include angiogenic, anti-apoptotic, mitogenic, and homing signals such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF) and SDF-1, resulting in the accumulation of several distinct populations of blood vessel progenitors and tissue-specific progenitor cells.[5, 7, 14-16] In the heart, paracrine secretion facilitates the recruitment of both cardiac progenitor cells and endothelial progenitor cells (EPCs). EPCs are essential to the initiation of neovascularization following ischemic injury. By comparing functional indicators after treatment with MSCs or MSCs engineered to overexpress SDF-1, Zhang and colleagues (2007) have directly shown that MSC-secreted SDF-1 provides trophic support to resident cardiomyocytes in the heart following myocardial infarction.[17] MSC engraftment has also been shown to raise the levels of endogenously produced bioactive factors. Using a stroke model in rats, Chen et al. (2003)[18] showed that IV infusion of human MSCs resulted in increased production of rat VEGF, with concomitant increased angiogenesis within the boundary zone of the ischemic lesion. In a separate investigation, the same research group observed an increase in endogenous neural cell proliferation.[16] This finding was accompanied by documentation of specific functional improvements with MSC treatment, including significant behavioral recovery at 7 days post-injury in a somatosensory test (adhesive removal) and at 14 days post-injury in a motor test (rotarod), compared to infusion of vehicle alone.[16]

Although the anti-inflammatory and paracrine effects of MSCs on the facilitation of wound healing have been well documented, much interest remains in research directed at evaluating the role of MSCs, as a stem cell population, to contribute to wound repair by direct differentiation to replace cells lost to injury. MSC differentiation to form cardiomyocytes, for example, has been demonstrated using both *in vitro* and *in vivo* methods.[19] *In vitro*, exposure to 5-azacytidine or retinoic acid results in increased expression of cardiac-specific transcription factors Nkx2.5 and GATA4 followed by the appearance of cardiac-specific proteins, including cardiac myosin heavy chain, α-sarcomeric actinin, phospholamban, and cardiac troponin T. *In vivo*, engrafted MSCs not only express a variety of cardiomyocyte markers, but have also been shown to establish intercellular connections with host cardiomyocytes through Cx43-positive gap junctions, revealing a direct contribution to the reconstruction of functional cardiac muscle through tissue-specific stem cell differentiation.[20] Moreover, MSC infusion to treat ischemic heart injury has suggested significant functional improvement in animal test subjects, including increased vascular perfusion and reduced fibrosis (reviewed in Pittenger *et al.*, 2004[1]). Positive results in efficacy measures have continued to hold up during the translation of MSC therapy to clinical investigation in heart attack patients.[21]

MSC homing and microenvironment-directed differentiation has likewise been noted in preclinical models of chemically induced lung damage. Ortiz and colleagues (2003) found a 23-fold increase in engraftment to the lungs of bleomycin-exposed vs. non-exposed control subjects.[22] IV-infused MSCs integrated specifically within sites of tissue injury and differentiated to form pneumocytes. MSC engraftment was associated with a significant decrease in inflammation, a reduction in collagen deposition and MSC differentiation to an epithelial phenotype in this and later evaluations of the therapy for the treatment of lung injury.[23-25]

Battlefield injuries commonly involve cutaneous tissue damage. MSC efficacy in promoting skin regeneration has been shown in multiple preclinical injury models, including thermal burn,[26-29] laceration[30-31] and radiation exposure.[32-33] Shumakov *et al.* (2003)[27] and others[28-29] have used thermal burn models to investigate the efficacy of topically applied or IV-infused MSCs to promote skin repair after injury induction. MSC administration decreases cell infiltration and accelerates the appearance of granulation tissue and new blood vessel formation in the wound. MSCs persist within the wound site during healing, resulting in more rapid wound closure. Evidence that MSCs play a natural role in the process of skin regeneration in humans has been collected in a clinical study in which the number of MSCs circulating in the peripheral blood of thermal burn patients was quantified and compared to the number of circulating MSCs in the blood of healthy volunteers.[26] MSC phenotype was determined by positive expression of 5 cell surface markers and negative expression of 8 markers. The percentage of MSCs in circulating blood was over 20-fold greater in burn patients compared to that of healthy individuals, and the degree of increase was correlated with the size and severity of the burn. These results offer data from human subjects suggesting that MSCs play an important role in skin regenerative processes, since the cells appear to be mobilized from the bone marrow in response to injury.

Several rat models have been utilized to study the ability of MSC therapy to facilitate wound closure following lacerative injury. In one study by Satoh *et al.* (2004), MSCs were injected intradermally into the skin of rats, and linear full-thickness incisional wounds were immediately made through the injected area. At 14 days post-incision, MSC-transplanted wounds had healed with very fine scars. Collagen architecture was thick, with an appearance similar to normal dermis. Histomorphologic scale analysis demonstrated a significant improvement in healing in MSC transplanted wounds compared to control wounds.[31] These findings have been supported by later studies in which MSCs were delivered by direct injection to the wound site,[34] as well as by IV infusion.[35]

A radiation-induced cutaneous injury model was utilized by Francois *et al.* (2007) to examine the potential of human MSCs to facilitate skin regeneration in a preclinical setting.[33] In this study, immune compromised mice received local irradiation (30Gy) to the leg and were infused with human MSCs 24 hours later. At 3 to 4 weeks, MSC treatment resulted in injury characterized by moist desquamation, while ulcerative lesions were observed in control mice. Increased healing rates were also observed in MSC-treated versus control mice, with histological examination of irradiated tissues

showing near complete healing of the dermis in MSC-treated mice at 8 weeks post-irradiation. Untreated mice showed only partial healing of the lesion at the same time point. Evidence that MSCs can participate in skin regeneration following radiation injury was also reported by Deng *et al.* (2005).[32] In this study, fluorescence-labeled MSCs from white (BALB/c) mice were infused to whole body irradiated (8.5 Gy) black (C57BL/6) mice. Fluorescently labeled MSCs were found to migrate and take residency within the skin, and black recipient mice grew white hairs that tended to spread over the body surface.

In addition to MSC efficacy in treating heart, brain, lung and skin, the promotion of both structural and functional improvement with therapy to treat a variety of other injury types has been described. For example, preclinical injury models have suggested MSC efficacy in tissue repair of the cornea,[36-38] liver,[39-40] kidneys,[41-43] skeletal muscle,[44] bone,[45-46] and tendon.[47-48] The homing ability and adaptability of cellular therapy to conditions local to the injury site make this approach ideal for medical defense, particularly in cases where more than one injury site or injury type is involved. The efforts reported here represent work being carried out at the United States Army Medical Research Institute of Chemical Defense (USAMRICD) with the intention of making this promising cellular therapeutic available to health care providers responsible for treating the warfighter. A first logical step to be taken in achieving this goal is the development of reliable procedures for the production of pharmaceutical quantities of MSC product.

Critical aspects in the design of a successful MSC production strategy include the selection of conditions that (1) maintain MSC differentiation potential, (2) provide consistently adequate cell yields and (3) are economically feasible. MSCs are readily isolated from whole bone marrow by differential adhesion to tissue culture plastic. However, the tissue culture environment differs dramatically from conditions present in the bone marrow, including the absence of the native MSC extracellular matrix (ECM) milieu. Fibronectin (Fn) is one of the most abundant ECM proteins in the bone marrow. One possible improvement in production conditions would be to include Fn in MSC culture methodology by coating culture vessel surfaces with Fn prior to stem cell seeding. The presence of Fn in MSC culture would provide an environment that more closely mimics the endogenous MSC niche, and would theoretically promote overall cell health and retention of stem cell differentiation potential. A second major difference in MSC growth under standard culture conditions is that freshly plated bone marrow produces colonies of tightly packed MSCs by the second week in culture, whereas MSCs are found primarily as widely dispersed single cells in the bone marrow environment. One promising improvement in MSC culture practices would therefore be to actively maintain low cell density, even at this early stage of MSC culture expansion. Although the maintenance of low cell density is fairly easily achieved during later cell passages, no report has suggested the dispersion of cells from these original colonies formed by the initial plated bone marrow as a significant consideration in MSC production strategy.

We have established methods for cell culture expansion of MSCs that result in consistently high yields in production lots derived from both rat and rabbit bone marrow. Compared to more conventional cell expansion approaches, significant improvements in MSC propagation methods have been identified by our laboratory in the past year with regard to maintenance of cell morphology and potency. These advances have resulted from a production strategy that involves growth over Fn-coated surfaces and a culture passage schedule that ensures that cells do not experience high density confluency for significant periods of time at any stage of the production process. As described herein, our production methods now allow for the generation of one lot of high-quality, differentiation-capable MSCs in 16 days or less, a significant advance as compared to approaches used by others that require approximately 8 to 9 weeks.[4, 18, 49-53]

METHODS

Animals

All MSC evaluations described in this report were made possible due to the use of tissue sharing by obtaining bone marrow samples from control subjects on active USAMRICD animal care and use protocols. These experimental protocols were approved by the Institutional Animal Care and Use Committee at USAMRICD, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

Rat bone marrow was obtained from either Sprague-Dawley rats (Charles River) or from an inbred transgenic rat strain that constitutively expresses the eGFP gene (Rat Resource and Research Center, RRRC; strain designation: Lew-Tg(CAG-EGFP)ys).[54] All rats used for the isolation of bone marrow were 12 weeks old or younger. Rabbit bone marrow for production of one lot of MSCs from a 1-yr-old donor and one lot from a 3-month-old donor was obtained from New Zealand white rabbits (Charles River).

Culture Methods

MSCs were propagated in Nunclon delta flasks and multi-tray cell factories (T-80, Nunc # 178905; T-175, Nunc # 178883; 2-tray factory, Nunc # 167695; 4-tray factory, Nunc # 140004). Prior to plating for cell production runs, tissue culture plastic was coated with fibronectin (Fn, Invitrogen # 33016-015) at a concentration of 12.5ug/mL in 1x calcium- and magnesium- free phosphate-buffered saline (PBS; diluted from Sigma # 14200-075) at a volume of 75ul/cm^2. Protein was allowed to adhere to the plastic surface for at least 1 hr at 37°C, and culture vessels were rinsed twice with PBS prior to cell seeding. Rat cells were grown in medium containing 44.5% Alpha-MEM (Invitrogen # 12571-063), 44.5% HAM/F12 (Invitrogen # 11765-047), 10% fetal bovine serum (FBS; Stem Cell Technologies # 6471) and 1% penicillin/streptomycin solution (Invitrogen # 15070-063). Rabbit cells were grown in 89% DMEM, 10% fetal bovine serum and 1% penicillin/streptomycin solution.

To initiate cultures at passage 0 (P0), bone marrow was flushed using PBS from both femurs and tibiae from each donor after removal of bone ends, using a bone cutter for rat bones and a small hand saw for rabbit bones. Marrow was flushed using a 22 gage needle for rat bones and a 14 gage needle for rabbit bones. The flushed samples were pelleted by centrifugation at 500 x g for 10 min, and resuspended in growth medium. Marrow was plated out at a density of 80 cm^2 per rat donor and 525 cm^2 per rabbit donor. Two days after culture initiation, flasks were rinsed twice with PBS and fed with growth medium. MSCs are differentially adherent to tissue culture plastic, compared to other cell types found in the bone marrow. The rinse procedure at two days post-plating and subsequent medium changes facilitate the generation of a virtually pure MSC population by the end of P3.

P0 cultures were allowed to grow until loose MSC colonies were formed. Cells were passed from P0 to P1 prior to the formation of tight cellular packing in colony centers. The targeted loose colony morphology occurred at 4 to 6 days for rat MSCs and 7 to 9 days for rabbit MSCs. Cells were detached from tissue culture plastic by brief (0.5 to 1.5min) incubation in 0.25% trypsin (Invitrogen # 25200-056). Trypsinization was stopped by the addition of an equal volume of trypsin inhibitor (Invitrogen # R-007-100). Trypsin and trypsin inhibitor were removed from the cell suspension by centrifugation at 500 x g for 10 min, aspiration of the supernatant and resuspension of the pelleted cells in the appropriate culture growth medium. MSCs were passed to new culture vessels at a density of approximately 6,000 to 8,000 cells per cm^2. Successive passages were carried out using identical methods once cells reached 80% to 95% confluency. This approach minimizes the degree and length of time of cell-cell contact, while still allowing for high yields at harvest.

MSCs were prepared for frozen storage at P1 (to generate a bank for later expansion to P3) or were grown to P3 directly without an interim freezing step. Initial production lots (all runs shown in Table 2) were frozen in 90% FBS and 10% dimethylsulfoxide (DMSO, Sigma # D2650). For these frozen lots, cells were resuspended in FBS prior to the drop wise addition of DMSO. Cryopreservation was initiated in a “Mr. Frosty” freezing apparatus (Sigma # C1562), containing 100% isopropanol in the outer insulation chamber, for 16 to 24 hr at -80°C. Cryovials were then transferred to the vapor phase of liquid nitrogen (-150°C) for long-term storage.

To test the growth of rat MSCs over Fn-coated tissue culture plastic versus uncoated plastic, the same methods described above for the growth of rat cells was used, except that the starting material for the test was generated from P0 cells that were not grown over Fn-coated plastic. Fn-coated and non-coated samples were then grown in parallel from P1 to P3 according to the same methods described for generating production lots, with the only variable being the presence or absence of Fn on the tissue culture surface.

Differentiation Induction

For the *in vitro* induction of bone differentiation, MSCs were incubated in osteogenic-inducing supplements (Chemicon # SCR028) and stained for alkaline phosphatase activity (Sigma # 85L3R) or calcium deposition by Alizarin Red staining (Millipore # 2003999), according to manufacturer’s instructions. Briefly, bone differentiation was induced by growth in 0.1 µM dexamethasone, 0.2 mM ascorbic acid, 10 mM glycerol 2-phosphate, and 10% FBS in the basal medium normally utilized for growth (HAM/F12 and Alpha-MEM for rat MSCs or DMEM for rabbit MSCs). *In vitro* adipogenic differentiation was induced by the addition of basal medium containing 10% FBS, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 µg/mL insulin, and 100 µM indomethacin. After 7 to 10 days, cells were cultured through one round of maintenance medium (10 µg/mL insulin and 10% FBS in basal medium) before returning to growth in induction medium. Adipogenic induction medium and

maintenance medium were prepared from components of the Chemicon kit # SCR020. The lipid vacuoles formed during adipogenic induction proved to be highly fragile, requiring extreme care during medium changes to avoid destruction of the cell monolayer. MSC-derived adipocytes were stained with Oil Red O (Millipore # 90358) or LipidTOX Red (Invitrogen # H34476) according to the manufacturer's instructions. For both osteogenic and adipogenic differentiation induction, cells were seeded at a density of 110,000 to 150,000 cells per well in a 12-well plate (Nunc # 150628) and fed every 2 to 3 days with the appropriate fresh medium. Cell morphology indicated the onset of differentiation after approximately one week for both differentiation regimens. Differentiation was allowed to continue for approximately 10 to 14 days for osteogenic induction and 2 to 3 weeks for adipogenic induction.

Microscopy and Image Capture

Cell culture growth was regularly observed using an Olympus CKX41 microscope, with both bright field and phase contrast capability. Images were captured with a Canon Powershot G9 fitted to the scope. Green fluorescence from eGFP-expressing MSCs was observed and imaged using an Olympus BX51 fluorescence microscope equipped with an Olympus DP70 camera or an Olympus FSX fluorescence microscope. Red fluorescence from LipidTOX Red signal and phase contrast images from adipocyte-differentiated cells were imaged using the Olympus FSX.

Cryopreservation Media Tests

The relative recovery and viability of MSCs obtained following cryogenic storage was assessed for MSCs prepared for freeze down using "traditional/standard" methodology (resuspension of MSCs in FBS, followed by slow addition of DMSO to a final concentration of 10%) compared to MSCs prepared for frozen storage using one of three commercially available cryopreservation solutions. The products tested included EZ-CPZ (Incell # EZCPZ5-20), Syntha-Freeze (Invitrogen # R-005-50) and Recovery (Invitrogen # 12648-010). Since the manufacturer's instructions for all three products suggest direct re-suspension of pelleted cells in freezing medium, an additional control was included in these tests consisting of a 90% FBS/10% DMSO solution in which the cells were similarly resuspended directly from pelleted cells. Four cell counts each for live and dead cells were taken for all data points on each experimental run, including counts to determine the total number of cells frozen down per vial and the total number of cells recovered for each test sample. Counts were obtained by sampling cells resuspended in culture medium diluted 50% in trypan blue (ScienCell # 0203). Data was obtained from three independent experimental runs, and statistical differences were determined by one-way ANOVA and Tukey's post-hoc analyses.

RESULTS

Growth Comparison of MSCs Cultured Over Non-Coated vs. Fibronectin-Coated Plastic

A test of fibronectin (Fn) vessel coating was performed to directly compare MSC growth from the start of P1 to the end of P3 in the presence or absence of Fn coating over a tissue culture surface (Table 1). The cell source for this test was P0 rat MSCs that had been grown over uncoated tissue culture plastic for 7 days. Cells were harvested from the P0 culture, and an equal number of cells were plated to non-coated and to Fn-coated flasks. MSC cultures were expanded in three consecutive passages, allowing cells to grow to 80 to 95% confluency before harvest and re-plating. P1 MSC cultures growing over Fn-coated plastic were ready to pass after 2 days growth, while MSCs growing on uncoated plastic were not ready for passage from P1 to P2 until 4 days from the start of P1. Each additional passage for MSCs growing over a non-coated surface also required 4 days growth before the targeted level of confluency was reached, while MSCs grown on a Fn-coated surface were ready for passage after 3 days growth. The total time in culture from P1 to P3 was therefore 12 days for cells growing on non-coated plastic vs. 8 days for cells grown over Fn-coated plastic. Note that colony dispersion at a relatively early time point during P0 is likely to have contributed to the overall reduced total production time, since MSC passaging of cells over a non-coated surface required only 4 days per passage, as compared to the 14-day time interval reported by others, as discussed below.

	Days to 80% to 95% Confluency			Total Days- P1 Start to P3 Harvest
	P1	P2	P3	
Non-Coated Plastic	4	4	4	12
Fn-Coated Plastic	2	3	3	8

Table 1. Fibronectin-coating test results. Cells growing over non-coated and Fn-coated tissue culture plastic were propagated at each passage until 80% to 95% confluency was reached.

MSC Production Run Results and Cell Morphology During Growth

Due to the apparent benefit on growth rate that Fn-coating provided in the test described above, this parameter was adopted into methodology used in MSC production runs. Rat and rabbit MSCs were grown from freshly harvested bone marrow to P3 before final harvest and cryopreservation, or to P1 for freeze down to generate a bank of cells to be used for later cell culture expansion. Table 2 details the growth during culture and final yield results obtained in MSC production runs performed to date.

These results represent significant improvements with regard to time-to-passage and total run time over previously documented MSC production strategies. Published duration times for the growth of passage 0 cells tend to run from 10 to 14 days,[4, 49-50] but have been reported to be as long as 21 days.[18, 51-52] Subsequent passages reported by others routinely run for 14 days each.[18, 51, 53] By the end of passage 3, therefore, conventional methodology for cell culture expansion of MSCs requires approximately 8-9 weeks, whereas the strategy described in this report results in the generation of one lot of MSC test material in 16 days or fewer.

Bone Marrow Source Species	# Donors	Freeze Passage	P0 Plating Area (cm2) / P0 Days	P1 Plating Area (cm2) / P1 Days	P2 Plating Area (cm2) / P2 Days	P3 Plating Area (cm2) / P3 Days	Total Cell Harvest (millions)
Rat	1	P3	80 / 6	350 / 2	1050 / 2	3792 / 2	99
Rat	1	P3	80 / 4	350 / 3	1264 / 3	5056 / 2	89
Rat	4	P1	350 / 5	1050 / 3	n/a	n/a	27
Rabbit (Age- 1yr)	1	P3	525 / 9	525 / 2	1610 / 2	5040 / 3	107
Rabbit (Age- 3mo)	1	P3	525 / 7	700 / 2	2528 / 3	7584 / 2	194

Table 2. MSC production run results. All bone marrow from both femurs and both tibiae of a single donor was used as the starting material for each plating that was passed to P3 before freeze down. The production run in which rat MSCs were frozen at P1 was initiated using bone marrow from both femurs and both tibiae of 4 donors.

One role of the MSC cell population within the bone marrow is to contribute to the structure of the hematopoietic stem cell (HSC) environment and to provide trophic support for HSCs. Figure 1(A) shows the collection of cobblestone-shaped hematopoietic cells over a small MSC colony at two days after bone marrow plating. These multi-cell-type structures are typical of early MSC cultures. However, during later culture feeds and cell passages, phenotype heterogeneity of the cultures is reduced, as seen in panel B, showing culture appearance just prior to passage from P1 to P2, and in panel C, showing culture morphology at P3 just prior to harvesting for freeze down as MSC stock test material. Panel D shows cells that were grown to 100% confluency, demonstrating the fibroblastic morphology of MSCs and overall “swirled” appearance of this cell population in a culture that has been allowed to reach high density.

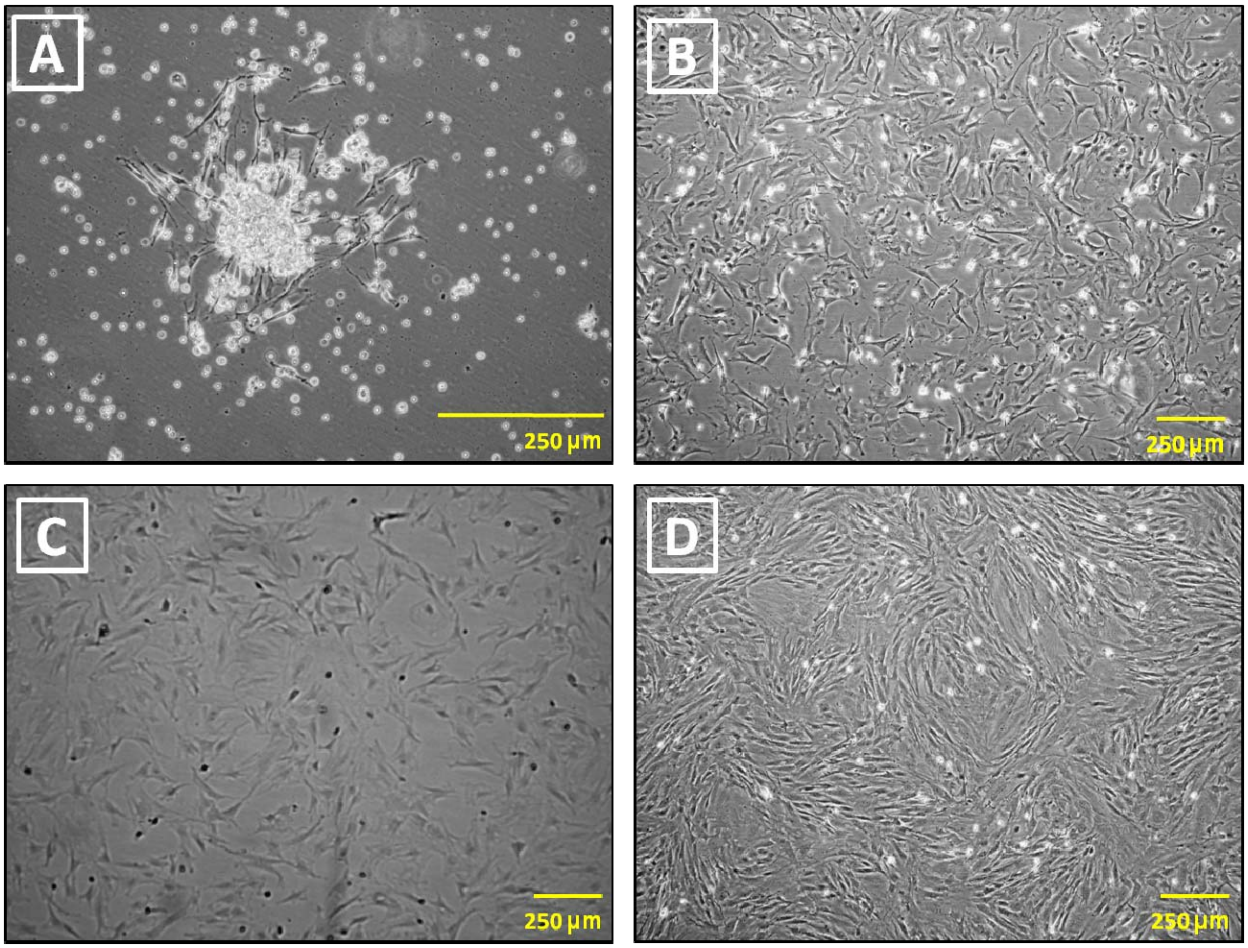

Figure 1. Rat MSC culture initiation, growth, and morphology during lot production. Bone marrow isolated from an eGFP transgenic rat strain was used to generate rat MSC cultured cells. (A) Initial growth of an MSC colony shown at day 2 after plating of whole bone marrow, showing hematopoietic cells collected at the center of the new colony. (B) P1 cultures prior to harvesting and plating for P2. (C) P3 cultures prior to harvesting and freeze down for preservation of frozen stocks (note: the poor quality of the image in this panel is the result of the cells being photographed through a 4-layer cell factory). (D) MSCs allowed to grow to confluency, showing the fibroblastic, spindle-shaped cell morphology and overall "swirled" organization typical of this cell population grown to high density.

eGFP Fluorescence and In Vitro *Differentiation of Rat MSCs*

All cells of the eGFP transgenic rat strain utilized in the work described here constitutively express the eGFP gene under control of the CAG promoter (a combination of the cytomegalovirus [CMV] early enhancer element and chicken beta-actin promoter).[55] The use of MSCs derived from this strain offers a significant advantage in that cells do not require the additional manipulation of cell loading with a

fluorescent cell marker for later detection during evaluations. eGFP fluorescence has been confirmed in eGFP rMSC production lots generated at USAMRICD, as shown in Figure 2, panels A (wide field) and B (close-up).

The differentiation capacity of rat MSCs obtained in various production lots was assessed through *in vitro* induction of adipogenesis and osteogenesis (Figure 2). Rat MSC adipogenic differentiation was documented by imaging both Oil Red O staining (C) and LipidTOX Red staining (D) of induced cultures. In C, the sample was counter-stained with hematoxylin for visualization of cell nuclei (blue/purple). In panel D, a red fluorescence channel image obtained from the LipidTOX red signal is overlaid onto a phase contrast image to show both lipid vacuole formation and overall cell structure within a single visual field. The formation of bone nodules in osteogenic-induced rat MSC cultures was demonstrated by detection of both alkaline phosphatase activity (E) and by calcium deposition (F).

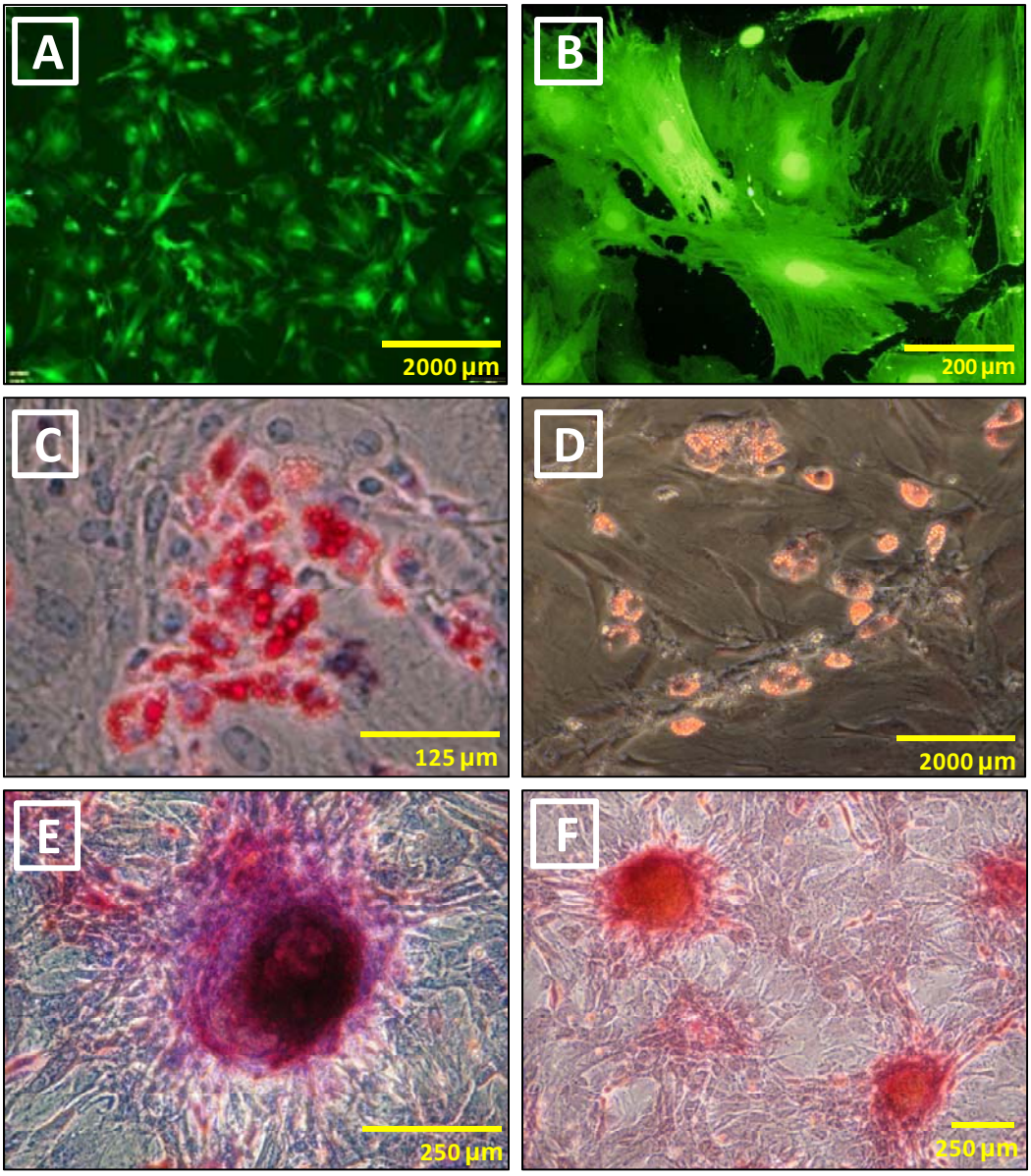

Figure 2. eGFP fluorescence and *in vitro* differentiation of rat MSCs. The fluorescent signal obtained from MSCs isolated from eGFP transgenic rats is shown in panels A and B. Green fluorescence from eGFP-expressing MSCs was imaged using a 485 nm excitation filter and 530 nm emission filter. Panels C and D are representative of MSCs cultured in the presence of adipogenic supplements. Adipogenic differentiation is shown in C by Oil Red O staining and in D by LipidTOX Red staining. Panel D represents an overlay of a phase contrast image and a 577 nm excitation/609 nm emission fluorescence image to capture the signal from LipidTOX Red. *In vitro* bone differentiation is shown in E by alkaline phosphatase activity and in F by staining for calcium deposition.

In Vitro *Differentiation of Rabbit MSCs*

MSCs isolated from rabbit bone marrow from a 1-year-old donor and a 3-month-old donor were evaluated for the ability to differentiate along the adipogenic and osteogenic lineages. Figure 3 shows representative outcomes for adipogenic differentiation induction of MSCs obtained from a 1-year-old donor (panel A) and 3-month-old donor (panel B) as assessed by Oil Red O staining. Panels C and D show calcium deposition in MSC cultures grown in the presence of osteogenic supplements for the same 1-year-old donor and 3-month-old donor, respectively. Both lipid vacuole formation of adipogenic-induced cultures and calcium deposition in osteogenic-induced cultures were found to proceed readily in MSC cultures obtained from the 3-month-old donor, while differentiation toward either pathway was highly limited in cultures of MSCs obtained from a donor that had reached 1 year of age.

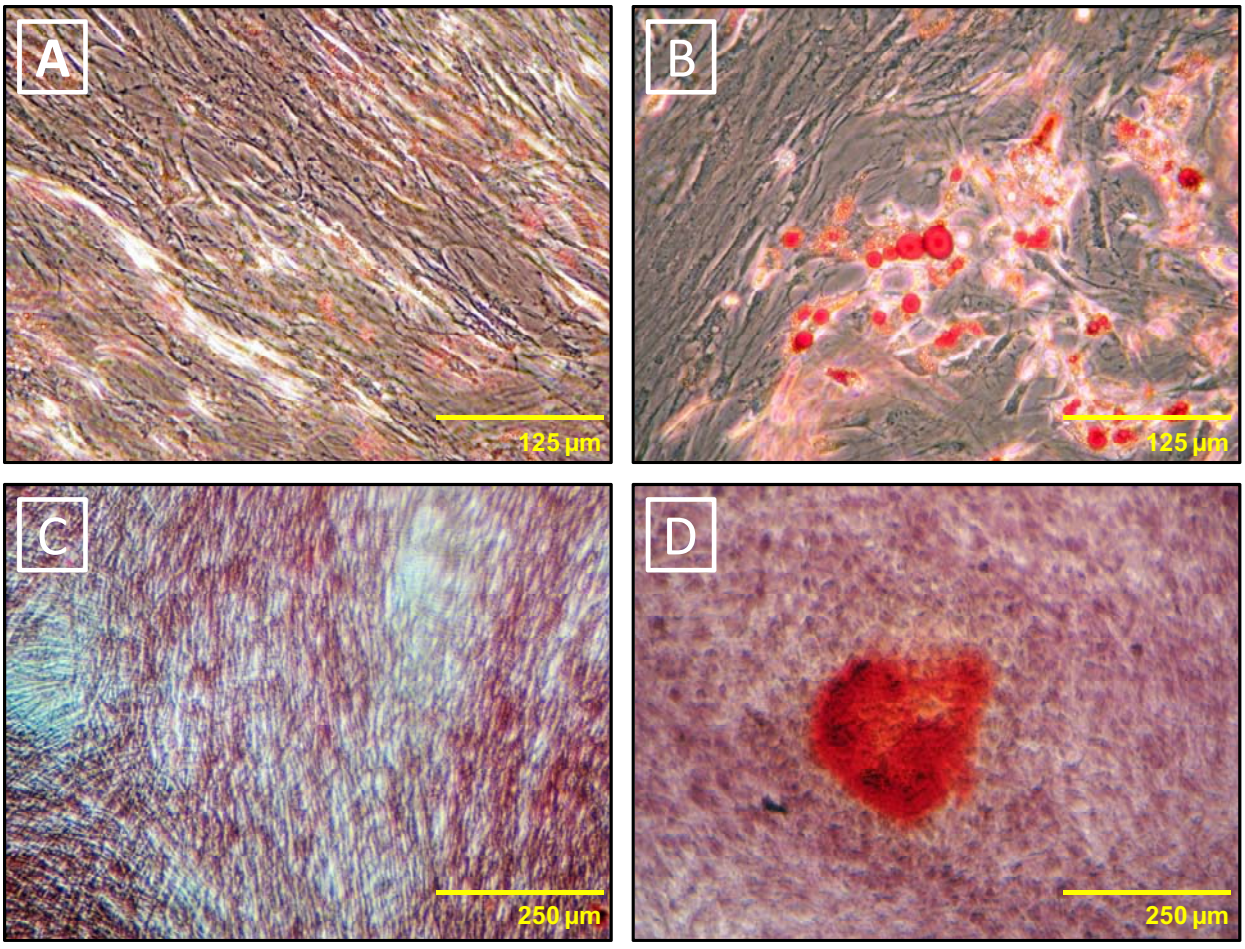

Figure 3. Donor age influence on MSC differentiation. Rabbit MSCs isolated from a 1-year-old rabbit and cultured in differentiation induction medium to stimulate adipogenesis (A) or osteogenesis (C) show minimal signs of differentiation as suggested by Oil Red O staining and calcium deposition staining, respectively. However, adipogenesis (B) and osteogenesis (D) were readily induced in MSC cultures

derived from a 3-month-old rabbit. Differentiation induction in MSC cultures derived from donors of each age group was performed in parallel.

Cryopreservation Media Test

As discussed above, initial production runs for rat and rabbit MSCs have consistently generated pharmaceutical quantities of MSC test material consisting of cells that have demonstrated the potential to differentiate to form mature cell types. One additional parameter of the MSC production process that has been evaluated is a comparison of "traditional/standard" cryopreservation techniques and reagents with newly available procedures and media. These tests were carried out with the goal of obtaining the highest possible level of recovery and viability of cells after thaw from frozen storage.

MSCs were harvested from growing cultures and were pelleted by centrifugation. Cells were prepared for frozen storage according to one of the following procedures: (1) resuspension in FBS at 90% of the final sample volume, followed by drop-wise addition of DMSO to reach 10% of the final volume ("DMSO-"), (2) resuspension in a pre-mixed solution containing 90% FBS/10% DMSO ("DMSO+"), (3) resuspension in EZ-CPZ, (4) resuspension in Syntha-Freeze, and (5) resuspension in Recovery. Figure 4 shows the results obtained from three independent evaluations carried out in parallel.

With regard to percent cell recovery, Syntha-Freeze and Recovery medium performed significantly better than cells processed for freezing using the "traditional/standard" method of adding DMSO to a final volume of 10% to cells previously suspended in FBS ($P < 0.001$ and $P < 0.05$, respectively). Also, the percent live cell recovery obtained from samples cryopreserved in Syntha-Freeze was significantly higher than that obtained with the EZ-CPZ product ($P < 0.05$). Percent cell viability upon thaw was lowest for "DMSO-" samples, with values for all remaining test samples at statistically higher levels ($P < 0.001$). Differences among samples for each of the two parameters tested were assessed using a one-way ANOVA test and Tukey's post-hoc analysis.

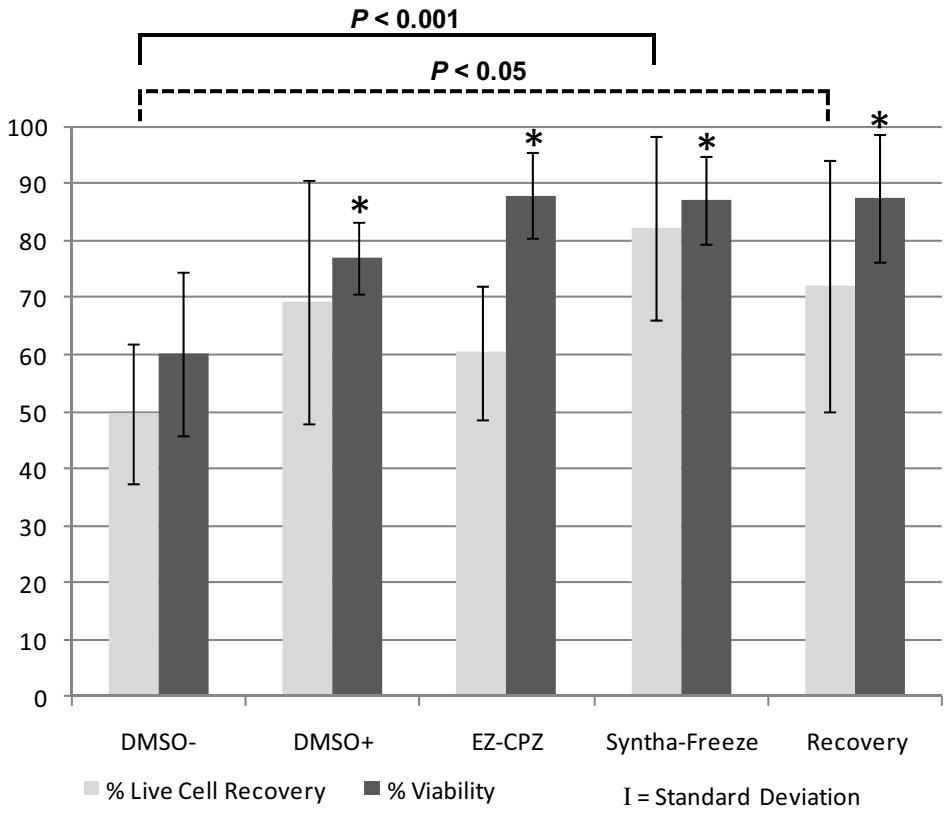

Figure 4. Cryopreservation Media Comparison. MSCs harvested from growing cultures were processed for frozen storage in one of five different cryopreservation medium types. For "DMSO-" samples, cells were resuspended in FBS prior to the addition of 10% DMSO. For "DMSO+" samples, pelleted samples were directly resuspended in 90% FBS/10% DMSO. All other samples were resuspended directly in the cryopreservation product indicated. Results shown were obtained from three independent evaluations performed in parallel. Bars represent standard deviation. Statistically higher values for post-thaw percent viability, compared to "DMSO-" samples ($P < 0.001$) are indicated by "*".

DISCUSSION

The dynamic nature of medical threats faced by the warfighter calls for the development of countermeasures that address a broad range of tissue pathology. The adaptability of the cellular therapy approach discussed in this report offers a promising option to address unmet needs of concern to medical defense. Current preclinical and clinical results of MSC efficacy investigations have demonstrated success in a variety of injury and disease settings.[36-47] Studies utilizing topical MSC application to treat readily accessible injuries, such as thermal burn, radiation burn and laceration have shown significant structural and functional improvements in wound healing. IV MSC delivery has shown efficacy at these sites as well and can also reach tissue damage located deeper within the body. This capability derives from the remarkable and well-

demonstrated behavior of IV-delivered MSCs to distribute to sites of compromised tissue under the influence of chemokine homing signals produced at one or more injury sites. The injury-specific distribution of MSCs following infusion offers a particular advantage in the treatment of combined injury (for example, laceration in combination with exposure to a chemical or radiological threat). IV MSC delivery represents an approach in which the administration of a single therapeutic can facilitate tissue repair at multiple injury sites and injury types, at a local level, in accordance with the specific requirements of each compromised site during the healing process.

MSC recruitment from the circulation depends on the expression of chemokines that promote MSC engraftment within sites of damaged tissue. A number of chemokines responsible for MSC recruitment are known to be involved in chemical injury. For example, USAMRICD scientists have clearly demonstrated MCP-1 and MIP-1α expression in the hippocampus, piriform cortex, and thalamus following soman (GD) exposure.[56] MCP-1 expression peaks at 24 hours after GD exposure, while MIP-1α levels are highest, depending on the specific brain region assayed, at between 12 and 48 hours. USAMRICD researchers have also demonstrated the upregulation of IL-8 secretion in human keratinocytes following exposure to the vesicating agent sulfur mustard.[57] The documentation that key chemokines responsible for MSC recruitment are induced in injuries resulting from chemical warfare agent exposure suggests a high likelihood that IV-infused MSCs will home to, and engraft within, sites of chemical agent injury.

The MSCs generated using our production strategy share the same canonical cellular morphology described by others, and we have demonstrated that these cells, when isolated from the bone marrow of relatively young donors, are capable of differentiating to produce mature cell types. The success we have gained in culture expansion of animal MSCs will guide the production methodology for human MSCs. Animal-derived and human MSCs will be generated for use in future *in vitro* and *in vivo* characterization and efficacy studies. The existing body of evidence that suggests the efficacy of MSC treatment over a wide range of injury types supports further investigation of the therapy as a candidate medical countermeasure. The data presented here demonstrate that we have developed a production strategy to generate pharmaceutical quantities of high quality MSC product, in a significantly shorter time frame than has been reported by others to date. The MSC culture expansion methodology described here can be used to produce test material for the treatment of injuries that are of critical concern to medical defense.

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